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13. ABSTRACT *(Maximum 200 Words)*

The long-range goal of the laboratory is to elucidate the role of genes involved in the development of breast and ovarian cancer, with a focus on the function of the tumor suppressor gene *BRCA1*. The human *BRCA1* gene codes for a nuclear phosphoprotein that is likely to be multifunctional. Recent genetic and biochemical evidence indicates that BRCA1 participates in the cellular DNA damage response. However, it is not known how BRCA1 exert its actions. Several lines of evidence suggest that it may function through transcription. Our working model is that impairment of BRCA1-mediated transcription activation predisposes to the development of cancer. By the end of the third year of our project we were able to develop a functional assay system to study the impact of all unclassified missense variants of BRCA1 that are located between aa 1366 and 1864 greatly expanding the scope of the transcription assay. In the course of this work we have also identified mutations in exon 13 that affect transcription activation and may define a region important for transcriptional regulation. Our results, taken together, reinforce the notion that sequences coded by exons 12 and 13 are important for the regulation of transcription by BRCA1.

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INTRODUCTION

The long-range goal of the laboratory is to elucidate the role of genes involved in the development of breast and ovarian cancer, with a focus on the function of the tumor suppressor gene *BRCA1*. Hereditary breast and ovarian cancer represent ~5% of all cases and inherited mutations in *BRCA1* account for the majority of breast and ovarian cancer families. The human *BRCA1* gene codes for a nuclear phosphoprotein that is likely to be multifunctional. Recent genetic and biochemical evidence indicates that BRCA1 participates in the cellular DNA damage response. However, it is not known how BRCA1 exert its actions. Several lines of evidence suggest that it may function through transcription. Our working model is that impairment of BRCA1-mediated transcription activation predisposes to the development of cancer.

In our original proposal we presented preliminary evidence suggesting a critical region for the modulation of BRCA1's transactivation activity. We proposed the existence of an intramolecular interaction domain that masks the activation domain of BRCA1, thus impairing the recruitment of the machinery involved in transcription initiation. Our hypothesis was that BRCA1 transcriptional function is regulated by an autoinhibitory intramolecular interaction between regions encoded by exon 12 (and possibly part of exon 13) and the C-terminal region comprising the BRCT domains. We suggested that this mechanism might form the basis of the regulation of transcriptional activation by BRCA1. Our expectation was that a region that interacts and masks the activation domain would be identified in detail and would reveal one of the means by which BRCA1 activity is regulated. In particular, it would be important to test whether germ-line mutations in BRCA1 that are suspected of cancer-association will modify this putative intramolecular interaction.

BODY

During the past year we focused mainly on performing experiments towards completion of Task 1, Task 2, Task 3 and initiating experiments described Task 4 and Task 5 described in our proposal and in the work statement (see below).

***Task 1.* To map the sites involved in the intramolecular interaction in BRCA1 using the yeast two-hybrid assay (months 1-12).**

- Express the tagged fragments in mammalian cells and check the interaction by immunoprecipitation and western blotting (e.g. IP with FLAG antibody and blot against GST antibody).

***Task 2.* To define the in vivo inhibition and the dominant negative activity of truncations of BRCA1 (months 8-24).**

- Optimize transient transfections of breast cancer cell lines and in ovarian cancer cell lines.
- Test if there is a difference in the *trans*-inhibition in breast cancer cell lines versus ovarian cancer cell lines.

***Task 3.* To analyze which mutations abolish transcriptional activation (months 12-24).**

- Introduce mutations found in patients with hereditary breast and ovarian cancer, especially those found in the putative interaction domain.
- Test how these mutations affect transcription activation of a reporter gene in yeast cells. If a particular mutation is found to modify BRCA1 activity, develop yeast two-hybrid constructs containing the mutations and test if it modifies the strength of interaction (judged by activation of β-galactosidase production in yeast two-hybrid assays.

Task 4. **To make deletions of the inhibitory domain (months 24-30).**

- Develop a series of plasmids for expressing deletions of the inhibitory domain in BRCA1 (defined in Task 1) in mammalian cells.

Task 5. **To map the phosphorylation sites involved in modulation (months 30-48).**

- Express different fragments of BRCA1 in mammalian cells spanning the region involved in the interaction using vectors that express tag epitopes (e.g. FLAG).
- Detect by in vivo labeling and immunoprecipitation with the tag antibody the fragments that are phosphorylated.
- Alternatively, phosphorylation can also be detected by observing a retardation of migration of the corresponding protein in SDS-PAGE.
- Define the putative phosphorylation sites by visual inspection of the sequence in the fragments that are phosphorylated in vivo.
- Introduce point mutations in the putative phosphorylation sites.
- Confirm that the mutant constructs fail to be phosphorylated in vivo.
- Develop GAL4 DBD fusion proteins of BRCA1 with disrupted phosphorylation sites.
- Test how these mutations affect transcriptional activation.

KEY RESEARCH ACCOMPLISHMENTS

- Development of a functional assay system to study the impact of all unclassified missense variants of BRCA1 that are located between aa 1366 and1864 greatly expanding the scope of the assay.
- Development of a structure-based algorithm to predict the outcome of all missense mutations in the BRCT region of BRCA1.
- Identified mutations in exon 13 that affect transcription activation and may define a region important for regulation.
- Determined that Serine to alanine mutations in the putative phosphorylation sites do not modify transcription activity in non-induced (non-irradiated) cells, thus allowing the use of these reagents to understand the role of phosphorylation in transcription activation.
- Identification in a mammalian two-hybrid system of a cDNA coding for Talin as a protein that interacts with BRCA1 sequences in exon 12 and 13.
- Identification and characterization of additional 11 temperature-sensitive mutants of BRCA1 in transcription activation.

PROGRESS

Progress on goals defined in Task 1.

We have performed several additional co-immunoprecipitation experiments using human embryonic kidney cells (293T) that show high endogenous levels of BRCA1. These cells were transfected with several constructs containing different fragments of BRCA1. We also used different lysis buffers of variable stringencies. In a series of transfections in which high levels of expression were obtained for the FLAG-tagged constructs (for a typical experiment see Figure 1; lane 1 393T cells transfected with FLAG-BRCA1 aa 1366-1454; lane 2 transfected with empty vectors), we were unable to immunoprecipitate full length BRCA1 (not shown).

We have also performed similar immunoprecipitations of lysates from cells cotransfected with a full length HA-epitope tagged BRCA1 and FLAG-tagged fragments of BRCA1. In addition the

amount of protein used in each immunoprecipitation was increased to 2 mg. Unfortunately, even with high expression levels of FLAG-tagged proteins, increased protein content per immunoprecipitations, use of different BRCA1 antibodies (Ab-1, Ab-2 and C-20), and less stringent binding conditions, the results obtained in this extensive series of experiments argue against our original hypothesis that there is an intramolecular interaction domain that binds the carboxy-terminal domain of BRCA1. However it is still possible that the affinity of the interaction is very low and cannot be detected in the conditions used. If this is the case, it should be evident in the experiments in Task 2. In addition, it is possible that the interaction epitope in the full-length protein is normally masked by interaction of sequences in *cis*. We are now planning to cotransfect fragments with different epitope tags (HA and FLAG) to perform immunoprecipitations.

All experiments proposed were performed and Task 1 is completed. *Nevertheless, we are performing some additional experiments to address the possibilities that were raised after the completion of the proposed experiments.*

Progress on goals defined in Task 2.

During the past year we have tested extensively the transfection of HCC1937, L56Br and MCF-7 derived from breast cancer and SKOV-3 and OVCar-2 derived from ovarian cancer. Several experiments were performed with these cell lines but with the current conditions we did not see a significant trans-inhibition by FLAG-tagged BRCA1 aa 1366-1718 nor by FLAG-tagged BRCA1 aa 1366-1559. This may be due to a level of expression that is significantly lower than the ones found in 293T cells. To circumvent this we are now testing the expression levels of the construct that has displayed the strongest *trans*-inhibitory effect, FLAG-tagged BRCA1 aa 1366-1454. In addition, if we cannot obtain significant levels of expression we will develop adenoviral vectors (which are already being used in the laboratory) to provide a much higher efficiency of transfection/transduction.

The first two series of experiments in Task 2 are completed. *The last two series remain to be performed due to technical difficulties.*

Progress on goals defined in Task 3.

After performing extensive analysis of transcriptional activation, we decided to embark on a stepwise approach to analyze the mutations (focusing on the ones that were suspected to be cancer-associated). Our initial idea was to adapt the functional assay and confirm that the assay was reliable with constructs with high activity (BRCA1 fragment comprising exons 13-24; aa 1396-1863 as shown in our original proposal). In this way we could analyze mutations were not included in our initial setup (aa 1560-1863)[1]. We then set up to analyze known cancer-associated mutations (M1775R; A1708E; Y1853X) and a known benign polymorphism (S1613G) to confirm their effect and also to test a number of other mutations thought to be important in the putative coiled coil domain. As shown in Figure 2, we confirmed that know cancer associated mutations were able to abolish transcription activation of a construct with very high activity. Most importantly, two mutations in exon 13 affected transcription activation. A construct with variant H1402Y was shown to be 3.5-fold more active than wild type BRCA1 in mammalian cells. Conversely, variant L1407P, which is predicted to cause disruption of the coiled-coil region, shows reduced activity. It is important to stress that is unusual for variants that are not in the BRCT domain to affect transcription by the carboxy-terminal of BRCA1. Taken together, the experiments in Task 3 also suggest an important role for sequences in exon 13 in the modulation of transcriptional activity by BRCA1. We are now in the process of subcloning variants H1402Y and L1407P into mammalian two-hybrid vectors to test their effect in the interaction with the full length and with several fragments of BRCA1.

With the exception of the last series of experiments all other proposed experiments were performed. Task 3 is partially completed.

Progress on goals defined in Task 4.

Our results suggest that sequences in exon 12 and 13 affect activation although it is still unclear how this regulation is exerted. Therefore we have obtained three constructs with deletions in full length BRCA1. Those are BRCA1Δ12, BRCA1Δ12/13 and BRCA1Δ13 that have a deletion in exon 12, a combined deletion of exon 12 and 13 and a deletion in exon 13, respectively. We are now in the process of testing their transcription activation activity in yeast cells.

Progress on goals defined in Task 5.

Several of the tasks described in Task 5, which involved the identification of putative phosphorylation sites, were superseded by the recent identification of phosphorylation sites of BRCA1. Several kinases involved in the early response to DNA damage have been shown to phosphorylate serine and threonine residues in BRCA1, including ATM, ATR and Chk2 (hCds1) [2-8]. Chk2 (hCds1) has been shown to target Ser988, a residue conserved in other Brca1 homologs [4]. Cortez et al. defined several residues to be a target for ATM both in vitro and in vivo following DNA damage (S1189, S1330, S1387, S1423, S1457, S1466, S1524 and 1542)[2]. Gatei et al. defined a more limited set of residues (S1387, S1423 and S1457). In addition ATR seems to target residue S1423 [5]. Since S988, S1189, S1330 are not included in our construct (aa 1366-1863) that contains our putative interaction site we did not analyze them at this point.

In order to narrow our focus on potential specific upstream kinases we introduced mutations (Ser to Ala substitutions) that disrupt specific known phosphorylation sites. We analyzed, in transcription activation assays, mutations in all putative sites identified in region aa 1366-1863 with special attention to residues S1423 and S1457 that seem to be the most physiologically relevant targets[2,3,7]. To rule out a direct effect on the overall activity independent of phosphorylation we initially tested the activity of the mutated BRCA1 in the absence of irradiation. This was done in yeast cells to evaluate the effect of the mutation on its transcription activity. Importantly, we analyzed protein levels to determine if there was an apparent effect of the mutation in the stability of protein.

We used the primers listed in Table 1 to introduce mutations by the quickchange method [1]. Presence of the mutation was confirmed by direct sequencing. The LexA DBD constructs containing single mutations were transformed into EGY48 cells and transcription activation was tested by β-galactosidase production. Our results (Table 1) suggest that the Ser to Ala mutations do not affect activation of transcription and therefore will be useful to analyze the role of phosphorylation since the artificial mutation *per se* does not affect transcription.

SUMMARY OF TASKS

- **Task 1** (Months 1-12): ***All experiments proposed were performed and Task 1 is completed.*** *Nevertheless, we are performing some additional experiments to address the possibilities that were raised after the completion of the proposed experiments.*
- **Task 2** (Months 8-24): ***Partially completed.***
- **Task 3** (Months 12-24): ***Partially completed.*** *Only the experiments described in the last item in Task 3 remains to be performed.*
- **Task 4** (Months 24-36): **Partially completed.**

- **Task 5** (Months 30-48): **All experiments proposed were performed and Task 5 is completed.** Nevertheless, we are performing some additional experiments to address the possibilities that were raised after the completion of the proposed experiments.

Table 1. Transcriptional activation by LexA-BRCA1 aa 1366-1863 containing phosphorylation site mutations.

LexA constructs	β-galactosidase activity (8 Lex operators)		
	30 min	2h	6h
Wildtype (positive control)	+	++	+++
S1423A	+	++	+++
S1457A	+	++	+++
S1497A	+	++	+++
S1524A	+	++	+++
S1542A	+	++	+++
M1775R (negative control)	-	-/+	+

Results were scored after X-gal addition.

ADDITIONAL ACHIEVEMENTS

We reported last year the identification of a temperature-sensitive allele of BRCA1. Given the fact that we had discovered its temperature-sensitive behavior in yeast transcription activation assays this raised the possibility that we could develop a mutagenesis screen to identify more mutants which such characteristics. Therefore, we followed the same procedure we had previously used to generate loss-of-function mutants in transcription activation [1]. Briefly, a cDNA fragment coding for the BRCA1 C-terminal region (aa 1560-1863) was subjected to PCR amplification using *Taq* polymerase, which due to its low fidelity, generates a number of mutations. PCR products were gel purified and co-transformed with a linearized vector containing a fusion of LexA DBD to wild-type BRCA1 (aa 1560-1863) and a β-galactosidase reporter responsive to LexA into yeast cells. In this process we took advantage of the highly efficient homologous recombination (gap repair) in yeast. We have identified several additional mutants that are described in the attached manuscript (that is currently being modified for resubmission to *Cancer Biology and Therapy*). In our opinion these mutations constitute important tools to dissect the role of BRCA1 in the DNA damage response and transcriptional activation.

Given the evidence suggesting a role for exons 12 and 13 in the regulation of transcription we decided to perform a yeast two-hybrid screening using a GAL4 DBD fusion to BRCA1 exons 12 and 13 in yeast as bait to screen a human mammary gland library. We isolated several double-positive (positive for growth in medium lacking histidine and β-galactosidase activity) clones. To narrow down the clones to be analyzed we decided to test the isolated clones in the more stringent mammalian two-hybrid system. The mammalian two-hybrid system may generate false negative results but no false positive results have been reported. We identified a cDNA clone coding for a region of Talin that interacts strongly with BRCA1 exons 12 and 13. We are now investigation this interaction using immunoprecipitation and in vitro binding assays.

REPORTABLE OUTCOMES

➢ Commentary Article on the function of BRCA1 in DNA damage response. *Cancer Biology and Therapy* (DoD support is acknowledged; copy attached).

➢ Article on the effects of missense mutations on transcription activation by BRCA1 (in preparation).

➢ Article on the characterization of temperature-sensitive mutants of BRCA1. Manuscript under revision to be resubmitted to *Cancer Biology and Therapy* (DoD support is acknowledged; copy is attached).

➢ Abstract submitted to Era of Hope (attached).

➢ **Funding from NIH RO1 with Total costs of 1,400,000 was obtained during the past year.**

➢ Funding for a postdoctoral fellowship from the New York State Board of Science and education in the laboratory was obtained during the past year for Claudia Lins-Bernardi.

CONCLUSIONS

By the end of the third year of our project we were able to achieve most of the goals defined in our proposal for this period as well as advance well into Task 5. We do not anticipate any problem in achieving all our proposed technical goals by the end of the 4th year of this project. We have made significant progress towards testing our hypothesis, in particular generating important reagents and optimizing conditions ideal to perform the experiments. At this point, our biochemical experiments designed to define an interaction domain have not revealed a clear picture. Although the experiments taken together reinforce the notion that sequences coded by exon 12 and 13 is important for transcription regulation.

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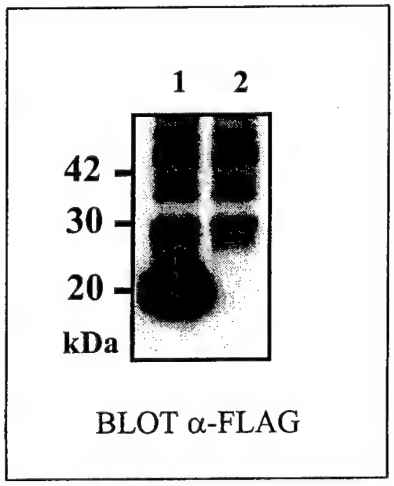

Figure 1

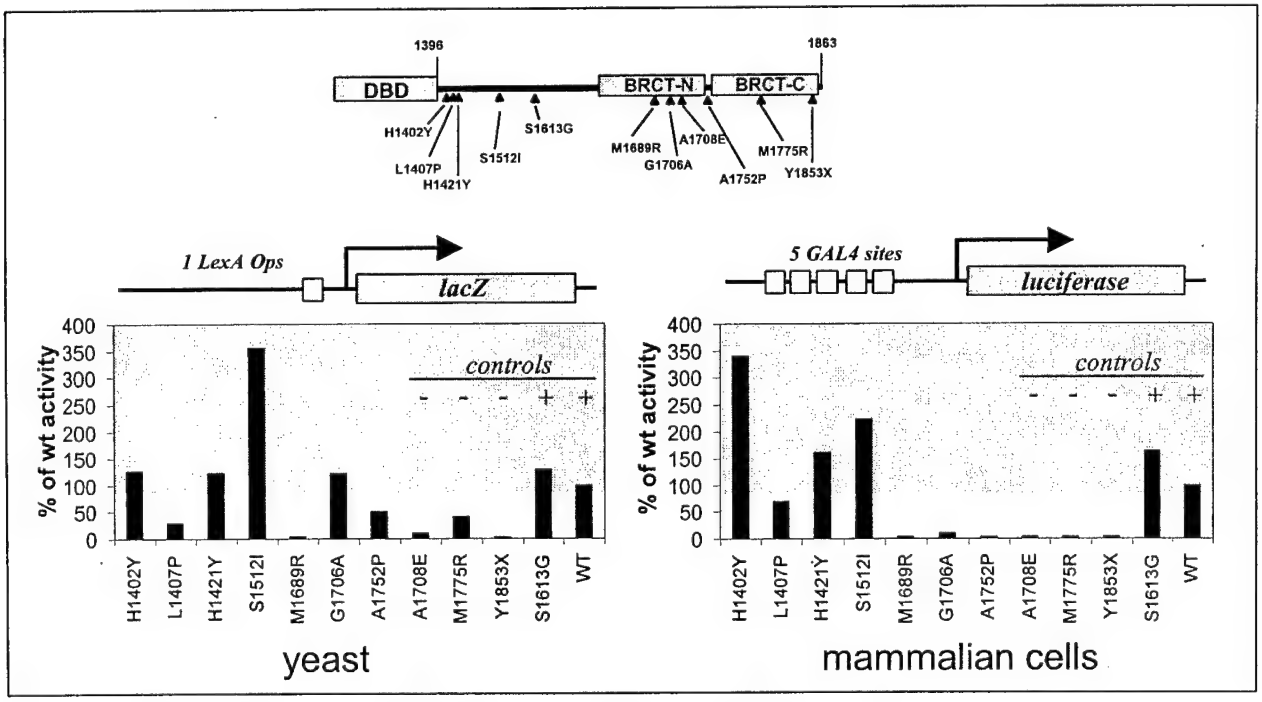

Figure 2

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Commentary

Participation of BRCA1 in the DNA Repair Response...Via Transcription

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Hereditary cancer syndromes, which usually represent a small portion of all cases, provide a genetically defined experimental system to understand the more common sporadic cases. For this reason the function of the breast and ovarian cancer susceptibility gene BRCA1 has been intensively pursued since its cloning and characterization in 1994.[1] Several lines of evidence indicate that its primary role is in the response to DNA damage but its precise biochemical function remains elusive (for a review see ref. 2).

BRCA1 has been implicated in a broad array of biochemical activities, some of them inferred by its association with other proteins of known function. These activities include transcriptional activation and repression, control of cell cycle checkpoints, participation in homology-directed recombination, transcription-coupled repair and ubiquitin ligase activity. Importantly, BRCA1 has been shown to be hyperphosphorylated in response to a wide variety of insults to DNA such as ionizing and UV radiation, hydroxyurea, hydrogen peroxide as well as several chemotherapeutic drugs. A conservative way of interpreting the current evidence is that BRCA1 is a link between the sensor and effector components of the response of DNA damage in mammalian cells. The finding that BRCA1 is recruited to sites of DNA damage earlier than Rad50 or Rad51 complexes suggest a role for BRCA1 in initial events following DNA damage.[3] One possible functional link is likely to be the regulation of transcription of genes encoding proteins that participate in the DNA damage response, including DNA repair. Consistent with this idea, the carboxy terminal domain of BRCA1 is capable of activating transcription and of interacting with the RNA polymerase II (for a review see ref. 4).

Early experiments demonstrated that BRCA1 cooperates with transcription factors such as p53 and STAT1 to induce the expression of the cell cycle inhibitor p21WAF1.[5-7] Array-based strategies later revealed that a major target for BRCA1 was the DNA damage responsive gene GADD45.[8,9] Interestingly, GADD45 was initially isolated as a gene induced by growth arrest and DNA damage agents.[10] Taken together these results suggested that BRCA1-mediated transcription would primarily guarantee a robust cell cycle arrest to allow the damaged cell enough time to repair its DNA.

Although there has been considerable interest in the response to ionizing radiation, only the initial events of BRCA1-mediated response to UV-induced damage have been studied. BRCA1 is phosphorylated after UV irradiation leading to relocalization of BRCA1 to PCNA+ complexes in S phase cells.[11] UV-induced phosphorylation is due primarily to the activity of the ATM and Rad3 related (ATR) kinase.[12,13] The downstream events and the biological implications of the ATR-mediated response are still unclear.

A new piece of the puzzle is provided by Takimoto et al[14] in this issue of Cancer Biology and Therapy. This study supports the role of BRCA1 in transcription activation and provides evidence that the transcriptional response to DNA damage mediated by BRCA1 is more pleiotropic than previously thought. In this study the authors show that BRCA1 collaborates with p53 to activate DDB2 following UV- and cisplatin-induced damage via a p53 responsive element present in the human DDB2 promoter. Although not essential for the p53-mediated transcription, the presence of BRCA1 enhances transcription activation of DDB2, the smaller subunit of the DDB heterodimer (composed of a 48 kDa and a 125 kDa protein). DDB binds to DNA damaged by UV or cisplatin and is mutated in a subset of patients with the cancer-prone syndrome xeroderma pigmentosum complementation group E.[15] Little is known about the function of DDB2 but recent evidence suggests a role in enhancing Global Genomic Repair of cyclobutane pyrimidine dimers.[16]

The study by Takimoto et al[14] also reinforces the connection between BRCA1 and p53. Besides the interaction of BRCA1 and p53 in transcriptional regulation, it has also been shown that inactivation of p53 partially rescues the embryonic lethal phenotype generated by disruption of Brca1 in the mouse.[17,18] Interestingly, inactivating mutations in p53 seem to be a hallmark of tumors arising in patients carrying germ-line mutations in BRCA1 or from Brca1-/-mice.[19,20] In addition, BRCA1 and p53 levels seem to be mutually

modulated.[21,22] These results indicate that a significant part of the biological actions of BRCA1 involve p53.

Takimoto and colleagues show that the BRCA1-mediated induction of DDB2 gene expression following UV irradiation is ATM-independent, consistent with previous reports that the UV response is mediated primarily by ATR. It will be interesting to confirm this prediction as well as to dissect the roles of the various phosphorylation sites in BRCA1. By the same token, it will be important to determine whether the induction of DDB2 by BRCA1 following Adriamycin exposure (resulting in DNA double strand breaks) is dependent on ATM. It has been proposed that different types of DNA damage signal to BRCA1 in different ways and it is conceivable that targeting different phosphorylation sites might direct BRCA1 into pathways geared towards the resolution of a particular type of damage. In this scenario, the role of BRCA1 would be akin to a routing platform adapting specific responses to the immediate needs of the damaged cell.

It has become increasingly clear that cells lacking BRCA1 are more susceptible to DNA damage and might prove to be a particularly sensitive to current clinical regimens such as irradiation or chemotherapy agents that generate DNA damage. However, the eventual success of this approach may depend on an intact apoptotic response. If cells with extensive DNA damage in which the apoptotic program has been inactivated fail to be eliminated these treatments may in turn promote the development of the tumor. For these reasons, a further dissection of BRCA1 role in transcription and in the participation in the DNA damage response is warranted and may reveal clinical avenues for future management of breast and ovarian cancer.

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MUTATIONS IN THE HYDROPHOBIC CORE OF THE BRCT DOMAIN CONFER TEMPERATURE SENSITIVITY TO BRCA1 IN TRANSCRIPTION ACTIVATION

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The breast and ovarian cancer susceptibility gene BRCA1 is a tumor suppressor and germ line mutations in this gene account for the majority of familial cases of breast and ovarian cancer. The precise biochemical function of BRCA1 is still unknown but there is mounting evidence indicating its involvement in two fundamental cellular processes: DNA repair and transcriptional regulation. A major hurdle to dissect the role of BRCA1 is the lack of molecular biological tools to carry out biochemical and genetic experiments.
To fill this gap we undertook a differential screen in yeast to isolate temperature-sensitive (TS) mutants of BRCA1 in transcription activation. Using a random mutagenesis approach we generated mutants of the carboxy-terminal region of BRCA1 (aa 1560-1863) that display a temperature-dependent activity in yeast transcription assays. We obtained 8 unique mutants that display wild-type activity in transcription at 30°C but markedly reduced activity at 37°C. In addition we were also able to identify 11 unique missense mutations that resulted in a loss-of-function phenotype at both temperatures. Mutations resulting in temperature-dependent activity were primarily located in the hydrophobic core of the BRCT domain of BRCA1. All TS mutants were subsequently analyzed in mammalian cells and showed loss of function at both temperatures suggesting that both the range of temperature and promoter stringency have to be adapted in order to generate TS mutants for mammalian cells. These conditional mutants will represent important tools to assess the role of BRCA1 in transcription in yeast and may form the basis to develop similar tools for mammalian cells.

Mutations in the BRCT Domain Confer Temperature Sensitivity to BRCA1 in Transcription Activation

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ABSTRACT

BRCA1 **is a tumor suppressor and germ line mutations account for the majority of familial cases of breast and ovarian cancer. There is mounting evidence that BRCA1 functions in DNA repair and transcriptional regulation. A major hurdle to dissect the role of BRCA1 is the lack of molecular tools to carry out biochemical and genetic experiments. Therefore, we used random mutagenesis of the C-terminus of BRCA1 (aa 1560-1863) to generate temperature-sensitive (TS) mutants in transcription activation. We obtained 11 TS mutants in transcription that localized primarily to the hydrophobic core of the BRCT-N domain of BRCA1. Analysis of TS mutants in human cells showed loss of function at both temperatures suggesting that the screening approach has to be adapted for mammalian cells. These conditional mutants represent important tools to assess the role of BRCA1 in transcription in yeast and may form the basis to develop similar tools for mammalian cells.**

INTRODUCTION

Germ-line mutations in *BRCA1* confer high risk for breast and ovarian cancer (Easton *et al.* 1993; Struewing *et al.* 1997). The molecular function of BRCA1 is not yet known but there is increasing evidence that it is involved in DNA damage and gene transcription (Monteiro 2000; Venkitaraman 2002). Several lines of evidence support a direct role for BRCA1 in transcription. When fused to a heterologous DNA binding domain (DBD) the C-terminus of BRCA1 activates transcription from a reporter gene and the introduction of cancer-associated mutations, but not benign polymorphisms, abolish activation (Monteiro *et al.* 1996; Chapman and Verma 1996; Monteiro *et al.* 1997). In addition, BRCA1 interacts with the RNA polymerase II and with several complexes involved in chromatin remodeling (Scully *et al.* 1997; Anderson *et al.* 1998; Bochar *et al.* 2000; Pao *et al.* 2000). Ectopic expression of BRCA1 results in the transcription of genes involved in cell cycle control and DNA damage repair (Somasundaram *et al.* 1997; Ouchi *et al.* 1998; Harkin *et al.* 1999; MacLachlan *et al.* 2000; Yarden *et al.* 2002; Takimoto *et al.* 2002). Interestingly, BRCA1 also interacts with CsTF50 in a complex that regulates mRNA processing pointing to a pleiotropic role in transcription (Kleiman and Manley 1999).

Despite the absence of *BRCA1* homologs in its genome, yeast has been an important model system to study BRCA1 as well as the function of several mammalian transcription factors (Kennedy 2002). Yeast has been utilized to perform structure-function analysis of BRCA1 in transcription as well as to probe its mechanisms of activation based on the correlation with the clinical data (Monteiro *et al.* 1997; Monteiro *et al.* 1996; Hayes *et al.* 2000; Vallon-Christersson *et al.* 2001; Nadeau *et al.* 2000). In

addition, overexpression of human BRCA1 in yeast generates a small colony phenotype that has been proposed as a method to classify uncharacterized mutations in BRCA1 (Humphrey *et al.* 1997). Thus, despite its limitations, yeast is a defined system to analyze BRCA1 function and is adequate for the rapid screening of large mutant libraries.

A major hurdle to define the function(s) of BRCA1 is the lack of molecular tools. Temperature-sensitive (TS) mutants would be particularly useful for this analysis. Recently, we have identified a *BRCA1* allele in a family with familial ovarian cancer that displays a temperature-sensitive phenotype (Vallon-Christersson *et al.* 2001; Worley *et al.*, in preparation). Therefore, we hypothesized that a differential screen in yeast based on random mutagenesis would allow us to isolate additional TS mutants. We followed the same procedure we had previously used to generate loss-of-function mutants in transcription activation (Hayes *et al.* 2000) and performed parallel screens at 30°C and 37°C. Here, we report the identification and characterization of 11 TS mutations in BRCA1 and 15 loss-of-function (LF) mutants. These mutants will allow the study of BRCA1 function in yeast and provide a basis for the development of conditional mutants for mammalian cells.

METHODS

Yeast. *Saccharomyces cerevisiae* strain EGY48 [*MATα, ura3, trp1, his3, 6 lexA* operator-*LEU2*] was co-transformed with the LexA fusion vectors and reporter plasmid pSH18-34 (Golemis 1994), which has *lacZ* under the control of 8 LexA operators (Estojak *et al.* 1995). The LexA DBD fusion of wild type human BRCA1 C-terminus (aa 1560-1863) and two germ-line mutants of BRCA1, Y1853X and M1775R were used as controls (Hayes *et al.* 2000). Competent yeast cells were obtained using the yeast transformation system (Clontech).

Error-prone PCR mutagenesis and screening. A 30-cycle PCR reaction (94°C denaturation; 55°C annealing; 72°C extension) was performed using *Taq* polymerase, p385-BRCA1 plasmid as a template and oligonucleotide primers (S9503101, 5'-CGGAATTCGAGGGAACCCCTTACCTG-3'; S9503098, 5'-GCGGATCCGTAGTGG CTGTGGGGGAT-3'). PCR products were gel purified and co-transformed in an equimolar ratio with an *NcoI*-linearized wild-type pLex9 BRCA1 (aa 1560-1863) plasmid and pSH18-34. Transformants carrying the mutagenized cDNAs were plated at 37°C or 30°C on plates lacking tryptophan and uracil and containing X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside), 2% Galactose, 1% Raffinose, X-Gal 80 mg/L, 1X BU salts (1L of 10X BU salts: 70g $Na_2HPO_4.7H_2O$, 30g NaH_2PO_4). The X-gal plates allowed direct visualization and were scored after 6 days. Clones were recovered from yeast and sequenced.

Mammalian cell reagents. A region comprising the BRCA1 coding region containing the TS mutation in pLex9 vector was excised with *Eco*R1 and *Bam*H1 and subcloned in pGBT9 in frame with GAL4 DBD. The fusion GAL4 DBD: BRCA1 was then cut with

*Hind*III and *Bam*H1 and ligated into pCDNA3. We used the reporter pG5E1bLuc, which contains a firefly luciferase gene under the control of five GAL4 binding sites (Seth *et al.* 1992) and transfections were normalized using a dual luciferase system (Promega). Human 293T and HeLa cells were cultured in DMEM supplemented with 10% calf serum and plated in 24-well plates at ~60% confluence the day before transfection. Transfections were carried out in triplicates using Fugene 6 (Roche, Indianapolis, IN) at 37°C for 2 hr. Cells were then incubated at 30°C or 37°C and harvested 24 hr post-transfection.

RESULTS

Screen for TS mutants of BRCA1 in transcriptional activation. We screened ~ 3 x 10^6 independent clones and recovered 1,302 putative LF mutants at 37°C (Figure 1A). These colonies were then plated on fresh plates and incubated at 37°C and 30°C for confirmation (Figure 1B). All plates contained yeast expressing wild-type cDNA to control for the different activity of β-galactosidase at both temperatures. Several clones turned out to display either a loss-of-function (white clones) or wild-type (blue clones) phenotype at both temperatures. Plasmids were recovered, retransformed into yeast and their activity confirmed. Clones that failed to display a reproducible activity were discarded. Plasmids representing 38 clones (3 were not recovered) were analyzed by restriction digest and although no clone had detectable deletions/insertions by gel analysis, sequencing revealed that 12 had nucleotide deletions or nonsense mutations and were not analyzed further. The remaining clones were processed for sequencing and the mutation identified. Eleven clones displayed markedly reduced activity at 37°C and wild-type activity at 30°C (TS clones; Table 1) and 15 had reduced activity at both temperatures (LF clones, Table 2).

TS mutants in yeast. Our screen resulted in the isolation of 11 TS mutants (8 unique) in transcription activation in yeast (Table 1). Seven clones displayed only one missense mutation and four clones displayed two missense mutations (Table 1). It is unclear whether the two mutations are required for the TS phenotype or not. At least in one case, TS32 (S1722F/K1667E), we know this is not the case because a similar mutation was found independently in another clone, TS25. Mutations causing TS activity were found in

exons 16-20 and 24. Interestingly, conserved hydrophobic residues were found to be a major target of mutations followed by mutations in serine residues (Table 1 and Fig. 1C). With two exceptions, L1639S and E1836G, all mutations occurred either in the N-terminal BRCT region or in the interval between the N- and C-terminal BRCTs (Table 1 and Fig. 1C).

Loss-of-function (LF) mutants. Due to the experimental design, several clones proved not to be TS mutants but instead LF mutants at both temperatures tested (Table 2). These mutations also targeted hydrophobic residues in the BRCT domains. Interestingly, we recovered a recurring cancer-associated mutation of BRCA1, M1775R (LF35; Table 2)(Friedman *et al.* 1994). Also, Q1811R and A1843P, found together in LF2, are unclassified variants listed in the Breast Cancer Information Core database (http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/). Two mutations, F1704S and L1657P, were found in 4 and 2 independent clones, respectively. Mutations causing LF phenotype were found in all exons examined with the exception of exon 19.

TS mutants in mammalian cells. All unique TS clones had their activity measured in mammalian cells using a fusion to GAL4 DBD and a luciferase reporter driven by a GAL4-responsive promoter. As negative controls we used two cancer-associated mutants, M1775R and Y1853X (Miki *et al.* 1994; Friedman *et al.* 1994). In four independent experiments none of the mutants reproducibly displayed significant activity (>30% of wild-type activity) at both temperatures in human 293T or HeLa (not shown). These results suggest that the requirements for temperature-dependent stability might be

more stringent in mammalian cells.

DISCUSSION

The function of BRCA1 has remained elusive despite extensive effort to characterize its biochemical activities. It has been implicated in DNA repair, transcription activation and repression, transcription-coupled repair, mRNA processing, cell cycle checkpoint and ubiquitination (Aprelikova *et al.* 1999; Monteiro 2000; Venkitaraman 2002; Baer and Ludwig 2002; Yarden *et al.* 2002). We reasoned that the isolation of conditional mutants would be an important addition in the experimental armamentarium to study BRCA1. Here we developed a screening strategy to isolate mutants of the BRCA1 C-terminus that display a TS phenotype.

We have isolated 8 unique TS mutants using this strategy. The fact that one mutation, F1734L, was found in 4 independent clones suggests that the screen might have reached saturation and that the mutants recovered reflect important regions for the regulation of BRCA1. In addition, two mutations, F1704S and L1657P, were found in 4 and 2 independent clones, respectively, in the loss-of-function set. If we consider that the screen has reached saturation we conclude that residues in the BRCT-N, in particular those located at its hydrophobic core (F1734L and H1686Q), are crucial to confer temperature sensitivity (see Fig. 1C) and therefore may be amenable to site-directed mutagenesis to design novel mutants. Interestingly, mutations in residues located at hydrophobic cores in the catalytic domain of tyrosine kinases as well as in SH3 domains have been demonstrated to confer temperature-sensitivity (Parrini and Mayer 1999). In three clones (TS1, TS4 and TS36) two mutations were found and only one of them may be important for temperature sensitivity. Alternatively, as found in TS mutants of v-Src, both mutations may be required (Mayer *et al.* 1986; Nishizawa *et al.* 1985).

Our screen also isolated 15 LF mutants (11 unique) and extended our analysis of mutants that cause loss of activity in transcription activation by BRCA1 allowing us to have a more detailed picture of the structure-function features of the C-terminal region of BRCA1 (Hayes *et al.* 2000; Vallon-Christersson *et al.* 2001). Again, we recovered mutations primarily in conserved hydrophobic residues but differently from the TS set those mutants were located not only at the BRCT-N but at the BRCT-C as well. It is important to stress that the TS mutants recovered are inactive at 37°C and are likely to represent cancer-associated variants if found as germ-line mutations.

The inability of these mutants to behave as TS mutants in mammalian cells may be due to inherent differences in the range of temperatures and metabolism of yeast versus the mammalian system. Alternatively, this may reflect the fact that the reporter used in the screen is not stringent. We tend to favor the latter explanation because there are documented examples of TS mutants isolated in yeast screens at 25°C and 33°C, permissive and restrictive temperature respectively, that turned out to display TS activity in mammalian cells at 34°C and 40.5°C (Parrini and Mayer 1999). This is a striking example in which the permissive temperature in mammalian cells was even higher than the restrictive temperature in yeast suggesting that the mutants adapt to the range of temperatures used in a particular host. The use of a low-stringency reporter is important at the restrictive temperature to guarantee the selection of mutants with the lowest possible activity. However, when screened at the permissive temperatures it will allow the selection of clones that may have low maximal activity. We are currently exploring these possibilities.

The clones isolated here are candidates to become molecular biological tools in yeast to dissect the function of BRCA1 in transcription and to guide further efforts to isolate TS mutants in mammalian cells. If we apply a conservative interpretation of the transcriptional assay, *i.e.* that it is simply a measure of the integrity of the BRCT domain, then it is possible that the data collected here may serve as a basis to rationally design conditional mutants to other proteins that present BRCT domains in their structure.

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Table 1. Temperature-sensitive mutants of BRCA1 (aa 1560-1863) in transcription.

Clone	Exon	Mutation	Nucleotide change[a]	Allowed residues[b]	Secondary structure[c]	Activity[d]	
						30°C	37°C
TS1	17	F1668S	T5122C	F	BRCT-N α-helix 1		
	24	E1836G	A5626G	DE	BRCT-C α-helix 3	+++	-
TS4	16	L1605L	T4932C	silent	unknown		
	17	V1687A	T5179C	V	BRCT-N β-sheet 3	+++	-
	19	K1727E	A5298G	KRQ	BRCT-N/BRCT-C interval		
TS6	16	L1639S	T5053C	LV	unknown	+++	-
TS19	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval		
	20	E1735E	A5324G	silent	BRCT-N/BRCT-C interval	+++	-
TS25	16	S1610S	T4949C	silent	unknown		
	19	S1722F	C5284T	S	BRCT-N α-helix 3	+++	-
TS26	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
TS30	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
TS32	17	K1667E	A5118G	KR	BRCT-N α-helix 1		
	19	S1722F	C5284T	S	BRCT-N α-helix 3	+++	-
TS33	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
TS36	16	S1631N	G5011A	SI	unknown		
	18	V1713A	T5257C	VI	BRCT-N β-sheet 4	+++	-
TS50	17	H1686Q	T5177A	H	BRCT-N β-sheet 3	+	-

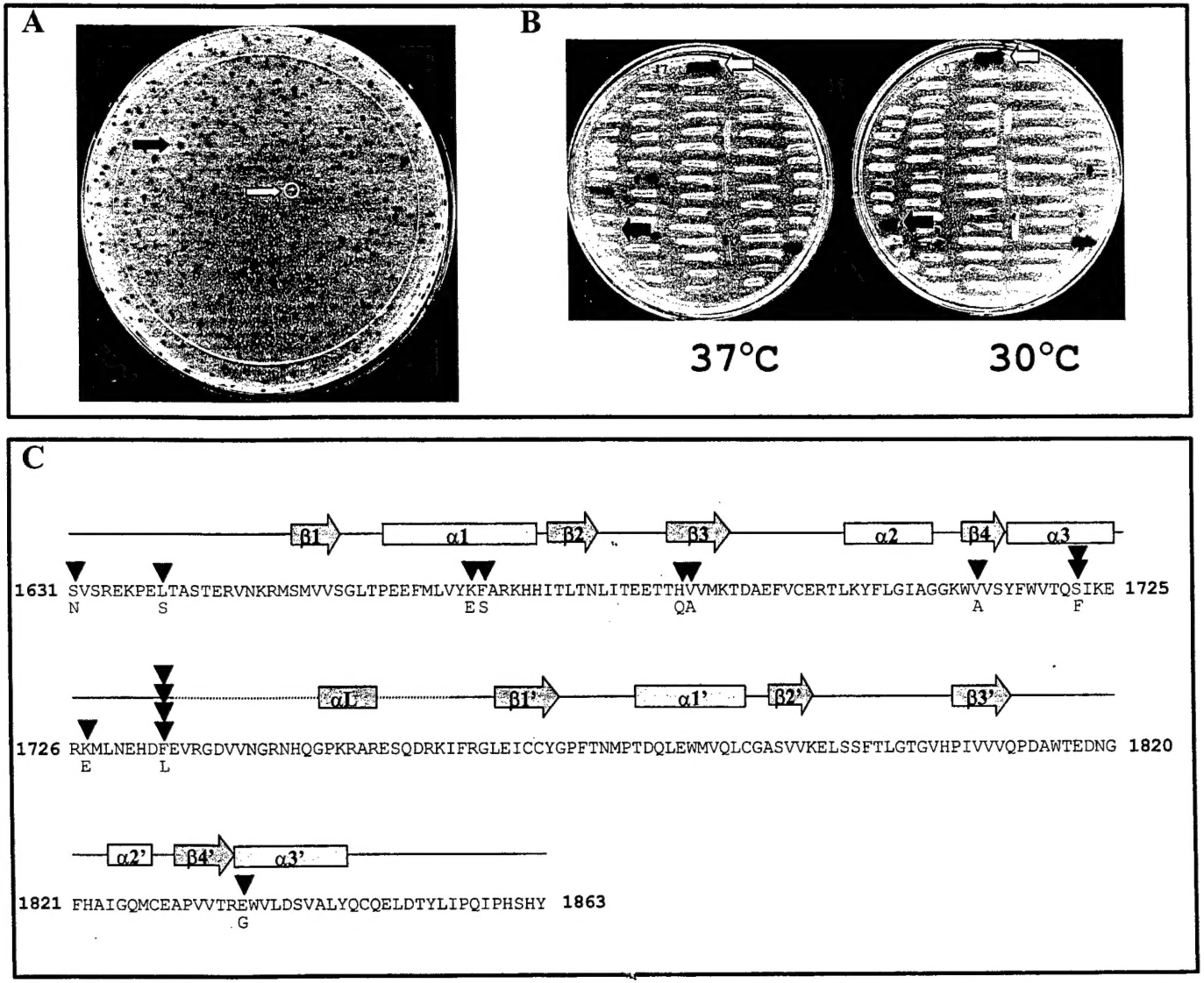
Table 2. Loss-of-function mutants of BRCA1 (aa 1560-1863) in transcription.

Clone	Exon	Mutation	Nucleotide change[a]	Allowed residues[b]	Secondary structure[c]	Activity[d]	
						30°C	37°C
LF2	23	Q1811R	A5551G	Q	BRCT-C β3-α2 loop		
	23	P1812S	C5553T	P	BRCT-C β3-α2 loop	-	-
	24	A1843P	G5646C	AS	BRCT-C α-helix 3		
LF3	17	L1671L	A5132G	silent			
	18	E1694G	A5201G	E	BRCT-N β3-α2 loop	-	-
	24	V1842A	T5644C	VIL	BRCT-C α-helix 3		
LF5	16	L1657P	T5089C	L	BRCT-N β1-α1 loop	-	-
LF8	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-
LF15	24	A1843T	G5646A	AS	BRCT-C α-helix 3	-	-
	17	T1691T	A5192G	silent	unknown		
LF20	17	F1668S	T5122C	F	BRCT-N α-helix 3		
	24	R1835R	A5624G	silent		-	-
	24	P1856T	C5685A	PQS	unknown		
LF22	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-
LF23	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-
LF24	20	G1743R	G5346A	G	BRCT-N/BRCT-C interval	-	-
	16	L1636L	T5034C	silent			
LF27	16	L1657P	T5089C	L	BRCT-N β1-α1 loop	-	-
	17	L1664L	C5110T	silent			
LF28	16	S1577P	T4848C	S --	unknown		
	16	S1655P	T5082C	S	BRCT-N β1-α1 loop	-	-
LF35	21	M1775R	T5443A	M	BRCT-C β1-α1 loop		
	22	Q1779Q	A5456G	silent		-	-
LF34	23	I1807S	T5539G	IVL	BRCT-C β-sheet 3		
	23	H1822H	T5585C	silent		-	-
LF38	16	E1660G	G5098A	EKSC	BRCT-N α-helix 1	-	-
LF47	16	R1649R	A5066G	silent			
	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-

[a] Nucleotide numbering corresponds to human *BRCA1* cDNA deposited in GenBank accession #U14680. Residues that are found in the same position in an alignment of human (U14680), chimpanzee (AF207822), dog (U50709), rat (AF036760), mouse (U68174), chicken (AF355273) and frog (AF416868) homologs. [c] According to the BRCA1 BRCT crystal structure (Williams *et al.* 2001). [d] Activity was scored in plates after 6 days. (+++) activity comparable to wild-type BRCA1.

LEGENDS

Figure 1. Temperature-sensitive mutants of BRCA1 in transcription. **A**. Primary screening at 37°C. Transformants carrying BRCA1 with wild-type activity appear as blue colonies (blue arrow) and transformants carrying loss-of-function mutants at 37°C appear as white colonies (white arrow). White colonies were replated in parallel and incubated at 30°C and 37°C. **B.** Plates containing replicas of each white clone isolated from primary plates. A transformant carrying a wild-type BRCA1 is included at the top of each plate (white arrow). Clones that were consistently white at 37°C and blue at 30°C were isolated as temperature-sensitive mutants (blue arrow). Clones that were white at both temperatures were isolated as loss-of-function mutants. **C.** Location of TS mutations in the BRCT region of human BRCA1. Secondary structure elements according to crystal structure of BRCA1 BRCT region (Williams *et al.* 2001) are depicted above the sequence. Interval region, separating BRCT-N and BRCT-C is represented by a dotted line with a α-helix (αL; purple). Residue positions mutated in TS clones are shown for clones containing one (red triangle) or two changes (blue triangle). Changes are indicated below the sequence.


A
B
37°C
30°C
C
β1
α1
β2
β3
α2
β4
α3
1631 SVSREKPELTASTERVNKRMSMVVSGLTPEEFMLVYKFARKHHITLTNLITEETTHVVMKTDAEFVCERTLKYFLGIAGGKWVVSYFWVTQSIKE 1725
N
S
ES
QA
A
F
αL
β1'
α1'
β2'
β3'
1726 RKMLNEHDFEVRGDVVNGRNHQGPKRARESQDRKIFRGLEICCYGPFTNMPTDQLEWMVQLCGASVVKELSSFTLGTGVHPIVVVQPDAWTEDNG 1820
E
L
α2'
β4'
α3'
1821 FHAIGQMCEAPVVTREWVLDSVALYQCQELDTYLIPQIPHSHY 1863
G